Previously uncharacterized rectangular bacteria in the dolphin mouth

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SUMMARY

Much remains to be explored regarding the diversity of host-associated microbes. Here, we report the discovery of microbial structures in the mouths of bottlenose dolphins that we refer to as rectangular cell-like units (RCUs). DNA staining revealed multiple paired bands that suggested cells in the act of dividing along the longitudinal axis. Deep sequencing of samples enriched in RCUs through micromanipulation indicated that the RCUs are bacterial and distinct from *Simonsiella*, a genus with somewhat similar morphology and division patterning found in oral cavities of animals. Cryogenic transmission electron microscopy and tomography showed that RCUs are composed of parallel membrane-bound segments, likely individual cells, encapsulated by an S-layer-like periodic surface covering. RCUs displayed pilus-like appendages protruding as bundles of multiple threads that extend parallel to each other, and splay out at the tips and/or intertwine, in stark contrast to all known types of bacterial pili that consist of single, hair-like structures. These observations highlight the diversity of novel microbial forms and lifestyles that await discovery and characterization using tools complementary to genomics such as microscopy.

KEYWORDS

dolphin, microbiota, bacterial morphology, rectangular morphology, pili, S-layer, microscopy, cryo-transmission electron microscopy (cryoEM), cryo-electron tomography (cryoET), single-cell genomics

INTRODUCTION

The earliest descriptions of the microbial world centered around the morphology and motility patterns of 'animalcules' (Leeuwenhoek, 1677). In the centuries since Leeuwenhoek's revolutionary advance, a vast diversity of microbial forms have been discovered, ranging from star-shaped bacteria in the *Stella* genus (Nikitin et al., 1966; Vasilyeva, 1985) to the multicellular fruiting bodies characteristic of Myxobacteria (Dworkin, 1999; Voelz and Reichenbach, 1969). Morphology is a biologically important characteristic, often highly conserved and molded over time by selective pressures resulting from an organism's lifestyle and environmental context (Young, 2006). Indeed, cell morphology plays an important role in motility, nutrient acquisition, cell division, and interactions with other cells, including symbioses with hosts, all of which are strong determinants of survival (Young, 2007). As such, morphological and structural studies offer an appealing route by which to glean insight into microbial life forms and the mechanisms by which species function and affect their environments. Moreover, characterizing the structures and functions of the diverse range of microbes in uncharted branches of the tree of life provides an opportunity to broaden our understanding of evolution and may result in myriad applications in biotechnology and medicine, exemplified by the development of optogenetics (Fenno et al., 2011) and CRISPR-based gene editing (Ishino et al., 2018).

Genomics serves as a powerful lens through which to describe the microbial world. In recent years, metagenomic and single-cell genomic analyses have substantially increased the number of known microbial phylum-level lineages, by a factor of nearly four in the bacterial domain (Anantharaman et al., 2016; Brown et al., 2015; Castelle et al., 2015; Rinke et al., 2013). Sequencing the genomes of newly discovered organisms has

73 led to the discovery of new functional systems, types of protein variants, and lifestyles
74 (Brown et al., 2015; Burstein et al., 2017; Donia et al., 2014; Dudek et al., 2017; Wrighton
75 et al., 2012), illustrating the correlation between phylogenetic diversity and functional
76 potential (Wu et al., 2009). However, the applicability of such approaches is mostly limited
77 to proteins and regulatory systems homologous to those of well-characterized organisms;
78 the prediction of phenotypes and functions that are truly novel and/or whose genetic basis
79 is unknown generally requires complementary knowledge. Given the recalcitrance of the
80 majority of the microbial species on Earth to laboratory culturing (Hug et al., 2016),
81 microscopy offers an appealing route by which to study novel morphological and
82 functional properties of uncultured lineages. Recent advances in cryo-electron
83 microscopy and tomography have allowed for intact bacterial cells to be imaged in three
84 dimensions at nanometer resolution (Pilhofer et al., 2010), leading to important advances
85 in the discovery and characterization of new microbial structures (Moissl et al., 2005;
86 Tocheva et al., 2010).
87
88 Despite the diversity of microbial cell shapes, rectangular structures are a rarity, with a
89 poorly understood genetic basis. Such structures are of two types: individual cells that
90 are rectangular, and cell aggregates that form rectangles. To the best of our knowledge,
91 the discovery of non-eukaryotic rectangular cells has thus far been restricted to the family
92 Halobacteriaceae, which consists of halophilic Archaea. Known rectangular cells from this
93 family include *Haloquadratum walsbyi* (Walsby, 1980), *Haloarcula quadrata* (Oren et al.,
94 1999), and members of the pleomorphic genus *Natronrubrum* (Xu et al., 1999). Additional
95 rectangular cells believed to be bacterial or archaeal have been discovered in high salinity
96 environments but not taxonomically identified (Alam et al., 1984). Amongst eukaryotic
97 microorganisms, diatoms can have a rectangular appearance (at least when visualized in

two dimensions), although these cells are cylindrical rather than rectangular prisms (Horner, 2002). A variety of bacteria form rectangular cell clusters, such as sheets of coccoid bacteria (for example, *Thiopedia rosea* and the genus *Merismopedia* (Zinder and Dworkin, 2006)), cuboidal structures of coccoid bacteria (for example, the genera *Sarcina* and *Eucapsis* (Zinder and Dworkin, 2006)), rectangular chains of filamentous bacteria (for example, the genus *Simonsiella* (Hedlund and Kuhn, 2006)), and rectangular trichomes formed by disc-shaped bacteria (for example, *Oscillatoria limosa* and other cyanobacteria (Zinder and Dworkin, 2006)).

Also rare in the microbial world are cells that diverge from the typical pattern of cell division along a transverse axis. One spectacular example is the nematode symbiont *Robbea hypermnestra*, which divides along its longitudinal axis (Leisch et al., 2016). During division, *R. hypermnestra* cells form rectangles as they divide (as they are two adjacent cells) and form a protective sheath around the nematode. This division patterning is thought to preserve attachment to the host (Leisch et al., 2016). Similarly, members of the genus *Simonsiella* divide longitudinally, which is thought to help with adherence to human epithelial cells in the oral cavity (McCowan et al., 1979). Although both of these bacterial taxa are uncultivated, deep insights about their biology have been gleaned from microscopy-based studies. Such insight into the reproductive methods of diverse bacteria is essential for building a comprehensive understanding of cell biology.

Previous studies using 16S rRNA gene amplicon sequencing and genome-resolved metagenomics found that the mouths of bottlenose dolphins (*Tursiops truncatus*) host a rich collection of novel microbes and functional potential (Bik et al., 2016; Dudek et al.,

122 2017). This diversity includes representatives from poorly characterized bacterial and
123 archaeal phyla (Hug et al., 2016), some of which lack cultured representatives altogether
124 and whose biology is therefore particularly poorly understood. To investigate the
125 morphology and potential lifestyles of these novel microbial lineages, we surveyed
126 dolphin oral microbial communities using various microscopy techniques. We discovered
127 unusual rectangular cell-like units (RCUs) in dolphin oral samples and characterized their
128 cellular dimensions and DNA patterning using phase-contrast and fluorescence
129 microscopy. Regular bands of DNA suggested that the units are sheets of individual cells,
130 and metagenomic sequencing strongly suggested that they are bacterial. Using cryogenic
131 transmission electron microscopy (cryoTEM) and tomography (cryoET), we characterized
132 the envelope structure of RCUs and discovered previously unobserved surface features
133 such as heterogeneous bundles of appendages that protrude from the ends of RCUs and
134 splay out at the tips. These findings highlight the power of high-resolution microscopy for
135 exploring the nature of uncultivated microbes.
136
137
138 **RESULTS**
139
140 **Rectangular cells in the dolphin oral cavity are Gram-negative and contain multiple**
141 **bands of DNA**
142 We collected a total of 28 oral swab samples from the mouths of nine bottlenose dolphins
143 (*Tursiops truncatus*) during two distinct intervals in 2012 and 2018, under the purview of
144 the U.S. Navy Marine Mammal Program in San Diego Bay, California, USA (**Methods**).
145 In phase-contrast images, rectangular cell-like units (RCUs) were readily apparent in 20
146 of the 28 samples (**Figure 1A-E**), including at least one sample from each of the nine

147 dolphins (**Table S1**). The RCUs resembled rectangular “pancakes” (**Movie S1)** and
148 exhibited Gram-negative characteristics following Gram staining (**Figure 1F**). RCUs
149 contained multiple parallel bands of fluorescence with DAPI staining (**Figure 1B,C,D,E**).
150 In some RCUs, the neighboring DNA band pairs formed “H”-like shapes (**Figure 1G**, white
151 arrow), suggesting two rod-shaped cells in the process of division along a longitudinal
152 axis with the DNA bands undergoing segregation. RCUs clustered into two morphotypes
153 based on the length of the DAPI-stained bands. In the “long DAPI-band” morphotype
154 (**Figure 1B,C**), the individual DAPI-stained bands tended to be longer than their “short
155 DAPI-band” morphotype counterparts (**Figure 1D,E**); the nomenclature does not refer to
156 the number of bands per RCU). We segmented and quantified the dimensions for the two
157 morphotypes (**Figure 1H**, inset), and found that the “short DAPI-band” morphotype
158 exhibited smaller width and length compared to the “long DAPI-band” morphotype
159 (**Figure 1H**). The different morphotypes may represent different cell types or taxonomic
160 groups (for example, strains or species), cells in different stages of development, or cells
161 with altered shape in response to environmental conditions. We focused on cells with long
162 DAPI bands, as this morphotype was more prevalent in the dolphin oral samples.
163

164 **RCUs are likely bacterial and not members of the genus *Simonsiella***

165 Given the intriguing morphology of RCUs, we next sought to determine their taxonomy
166 and phylogenetic affiliations. RCUs bear qualitative resemblance to members of the
167 genus *Simonsiella*, which consist of rod-shaped bacteria that collectively form a
168 rectangular unit and are oral commensals in mammals (Hedlund and Kuhn, 2006). Thus,
169 we first sought to evaluate the likelihood that RCUs are members of this genus. In a re-
170 analysis of the Sanger clone library and 454 pyrosequencing data from a previous 16S
171 rRNA gene amplicon survey of gingival swab samples from 38 dolphins from the same

172 population (Bik et al., 2016), no *Simonsiella* amplicons were detected in any of these
173 samples. *Simonsiella* was detected in the Sanger library from the mouth and gastric fluid
174 of a sea lion examined in the same amplicon study, confirming that *Simonsiella* DNA can
175 be extracted successfully with the protocol used in this and the previous study (Bik et al.,
176 2016). We next selected three new dolphin oral samples that we visually confirmed to
177 contain RCUs and performed 16S rRNA amplicon sequencing (**Methods**), resulting in the
178 detection of 394 amplified sequence variants (ASVs) with 991,592 amplicon reads across
179 the three samples (**Figure 2**). Thirty-one ASVs from 21 taxa were shared among the three
180 dolphin oral samples and are thus potential RCU ASV candidates (**Table S2**). No
181 *Simonsiella* amplicons were detected in these three samples.
182
183 The marine origin and rectangular nature of the RCUs also gave rise to speculation that
184 they may be marine diatoms (for example, *Skeletonema costatum*), as cylindrical marine
185 diatoms may appear rectangular in two dimensions. Thus, we next sought evidence of
186 eukaryotic RCU origin. We performed fluorescence *in situ* hybridization (FISH) using
187 labeled eukaryotic (Euk-1209) and bacterial (Eub-338) probes, the latter of which is
188 known to hybridize with both bacteria and archaea. As controls, we cultured and included
189 *S*. *costatum* and the bacterium *Escherichia coli*. The Eub-338 probe hybridized to both *E.*
190 *coli* and the RCUs, while the Euk-1209 eukaryotic probe hybridized to *S. costatum* cells
191 alone (**Figure 3**), indicating that RCUs are not eukaryotic and thus not diatoms.
192
193 With no further *a priori* hypotheses as to the specific nature of the RCUs, we pursued a
194 variety of general approaches to shed light on their identity. First, we cultured oral
195 samples under aerobic and anaerobic conditions in three different media used to grow
196 diverse bacteria (Methods), hoping to enrich for RCUs. Unfortunately, RCUs were not

197 visible upon inspection of cultures under a microscope, indicating that the growth
198 requirements for RCUs are distinct from those for typical bacteria isolated from
199 mammalian microbiotas.
200
201 Our next strategy employed single-cell genomics. Notably, this approach largely avoids
202 preconceived biases about possible identity, since single-cell genomics should detect
203 DNA from any cell from any domain of life, assuming successful cell lysis. We used three
204 techniques to capture rectangular cells for genomic sequencing: laser capture
205 microdissection, microfluidics, and cell micromanipulation. Due to their large size
206 compared with other bacteria, low density, and the propensity of RCUs to stick to other
207 cells and to abiotic surfaces, only micromanipulation led to successful RCU capture
208 (**Figure S1**). In addition to four collection tubes each containing ~1-3 RCUs, four negative-
209 control tubes of sample fluid were collected with the micropipette without any cells visible
210 at the resolution of our microscope. Cell-free DNA and small non-target cells were also
211 likely collected along with the RCUs, given the frequent close proximity of RCUs to other
212 cells. DNA from RCU-positive and -negative samples was amplified using multiple
213 displacement amplification (MDA), co-assembled, and sorted into 18 genome bins
214 (**Figure 4**; **Figure S2; Tables S3, S4**; **Methods**). In addition to providing several
215 candidate identities for the RCUs, this experiment provided further evidence against the
216 RCUs being marine diatoms or *Simonsiella*, as no bins were assigned to either of these
217 taxonomic groups. The eukaryotic bins matched the human genome or the fungal class
218 Malasseziomycetes, members of which are known commensals of human skin (Limon et
219 al., 2017), and hence likely represent contaminants. No archaeal bins were recovered.
220

221 The lack of a consistent, shared, and abundant set of genome sequences (likely due to
222 contamination from different non-RCU cells and/or lack of recovery of RCU DNA from
223 positive samples) precluded a confident taxonomic identification of the RCUs. When we
224 excluded bins that were present in any negative control with ≥1% relative abundance (an
225 admittedly arbitrary threshold) and for which an ASV from the same taxonomic group was
226 detected in all three dolphin oral samples that underwent amplicon sequencing, six
227 candidate taxa remained: 1) phylum Bacteroidetes, genus *Tenacibaculum* (76%
228 complete); 2) phylum Epsilonproteobacteria, genus *Arcobacter* (76% complete); 3)
229 Epsilonproteobacteria, family Campylobacteraceae (14% complete); 4) phylum
230 Gammaproteobacteria, family Moraxellaceae (58% complete); 5) Gammaproteobacteria,
231 unknown family (0% complete, i.e. there were assembled scaffolds in the bin even though
232 they did not encode marker genes used to estimate completeness; **Figure S3**); and 6)
233 Gammaproteobacteria, unknown family (83% complete). The relatively well-studied
234 genera *Tenacibaculum* and *Arcobacter* are unlikely candidates for RCU taxonomy, and
235 are more likely to be contaminants. If each RCU were comprised of ~8 cells (segments)
236 and the co-assembly were based on samples from >4 RCUs, then the larger copy number
237 of RCU genomes should have resulted in better quality genome assembly, suggesting
238 that a true RCU bin should have greater completeness, suggesting general technical
239 challenges faced by genomic approaches.
240

241 **CryoEM reveals nanoscale surface and internal structures of RCUs**

242 To gain high-resolution structural insight into RCUs, we imaged dolphin oral samples
243 containing high densities of RCUs using cryogenic transmission electron microscopy
244 (cryoTEM). Low-magnification cryoTEM images revealed that each RCU consists of
245 seemingly paired segments organized in parallel (**Figure 5A,B**); these segments were

oriented similar to the DAPI-stained bands seen in fluorescence microscopy images (**Figure 1C,E**). Groups of segments sometimes appeared to be in the act of separating from other groups, although our static data cannot definitively say whether this appearance was reflective of cell division. Segments were surrounded by a dense, membrane-like layer under a low-density layer (**Figures 6A,B (right) and 7A; Movies S2, S3**).

We hypothesized that RCUs are most likely aggregates of cells, with each DNA-containing segment, an individual cell. The following observations support this hypothesis: 1) segments appeared to be surrounded as a group by surface structures reminiscent of plasma membranes and/or cell walls (**Figures 6A,B (right) and 7A**); 2) segments are arranged in the same geometry (**Figure 5A,B**) as the DAPI-stained bands and FISH probe-hybridized bands (**Figure 1C,E,G**); 3) appendages protruded from the surface of individual segments (**Figure 5C,D**) ; 4) RCUs often consisted of groups of variable numbers of segments that appeared to be separating from one another (**Figures 1D,E and 6B**), suggesting that the rectangular structures do not reflect an individual cell.

Dark, spherical structures that were visible in the body of the RCUs in cryoTEM images may be storage and/or lipid granules. In one tomogram, two dense spheroidal objects (**Figure 5C**) were prominently visible and measured 192 nm × 200 nm × 192 nm (volume 3.1×10^{7} nm^3) and 215 nm × 220 nm × 220 nm (volume 4.4×10^{7} nm^3). Vesicle-like structures were also apparent. Notably, a surface covering with a periodicity of ~7-9 nm encapsulated the RCUs (**Figures 6 and 7; Movies S2, S3**).

To obtain more detailed three-dimensional reconstructions of RCU features, we conducted cryoET experiments. Tilt-series acquisitions were limited to the RCU periphery since the thickness of the RCU bodies occluded the electron beam at high tilt angles. The thickness at the RCU periphery (<1 μm from the edge) ranged from ~323 nm to ~751 nm, with an average value of ~509 nm (*n*=15). Appendages that resembled pili (Hospenthal et al., 2017; Proft and Baker, 2009) protruded from RCUs; these appendages often consisted of parallel, hair-like structures that formed bundles and splayed out at the tips, sometimes intertwining and/or crossing over one another (**Figures 6B and 7B,C**). The bundles of appendages were structurally heterogeneous, with variable lengths, bundle widths, and numbers of tips. Notably, in examining the various features within the tomograms, we did not observe any membrane-bound organelles reminiscent of a nucleus, in line with a non-eukaryotic identity.

For both the appendages and periodic surface covering, subtomogram averaging (Galaz-Montoya and Ludtke, 2017) did not yield consistent maps, likely due to the thickness of the RCUs (often >500-600 nm thick), low signal-to-noise ratio of the tomograms, and intrinsic characteristics of the features in question, such as the high and variable curvature of the regions with a continuous periodic surface covering. These cell surface features, which are essential for a cell's interaction with its environment, merit future investigation as they defy the conventional wisdom that pili-like appendages are single, hair-like structures (Hospenthal et al., 2017; Proft and Baker, 2009), and that periodic surface coverings (such as S-layers) encapsulate a single cell (Fagan and Fairweather, 2014; Sleytr et al., 2007) rather than a collection of cells.

295 **DISCUSSION**

296 Here, we used optical microscopy, cryoTEM, and cryoET to search for novel
297 morphological diversity within the microbiota of dolphin oral samples, which was predicted
298 based on previous findings of novel phylogenetic diversity and functional potential in the
299 dolphin mouth via sequencing-based studies (Bik et al., 2016; Dudek et al., 2017).
300 Interestingly, we discovered morphologically unusual RCUs. We infer that they are
301 endemic to the dolphin mouth given that they were consistently present in this
302 environment: they were identified in 20 out of 28 samples investigated, including in at
303 least one sample from all nine dolphins included in this study, and were present in
304 samples collected during intervals six years apart. Previous studies have found that the
305 microbiota of marine mammals is distinct from that of seawater (even that of skin, which
306 is constantly in direct contact with seawater) (Apprill et al., 2011; Bik et al., 2016), and
307 thus it is unlikely that RCUs are simply contaminants from seawater.

308

309 The taxonomic identification of specific cell morphotypes from complex communities can
310 be extremely difficult, to the point that it often remains unresolved (Alam et al., 1984; Luef
311 et al., 2015; Wanger et al., 2008). Results from DAPI staining, cryo-EM imaging, FISH,
312 and single-cell genomics indicate that the RCUs are bacterial, and 16S rRNA amplicon
313 sequence-based surveys and single-cell genomics suggest that RCUs are distinct from
314 species in the genus *Simonsiella*, a bacterial taxon whose members also form
315 rectangularly-shaped clusters of rod-like cells. Obtaining a species-level identification via
316 sequencing-based methods will be extremely challenging for numerous reasons, such as
317 the frequent close proximity of RCUs with other small cells that were likely mixed with
318 RCUs during micromanipulation or recalcitrance to laboratory lysis. Importantly, we are
319 hesitant to exclude candidate identities based on not being present in all positive RCU

320 samples, since technical limitations could have resulted in false negatives. For example,
321 a thick cell wall or obstruction preventing reagents from reaching the RCU by the
322 micropipette needle could have interfered with lysis of the cell membrane and impeded
323 DNA extraction. Conversely, a positive result in a negative control may have arisen due
324 to non-specific read mapping, contamination of genome bins with material from true
325 contaminants, or cell-free DNA. Culturing-based approaches may ultimately be the most
326 promising route forward for identifying RCUs, although many combinations of parameters
327 will likely need to be tested to find satisfactory conditions for RCU growth. If RCUs are
328 strictly anaerobic, culturing may be further complicated since swabs cannot be collected
329 from the mouths of live dolphins without exposure to the atmosphere during the collection
330 process. Regardless of the taxonomic identity of the RCUs, the novel structural features
331 that have evolved in these microorganisms are intriguing and highlight the discovery
332 potential for further study.
333
334 The paired nature of segments in RCUs can likely be explained by longitudinal binary
335 fission behavior, as seen in members of the *Simonsiella* genus and *R. hypermnestra*
336 (Leisch et al., 2016; Steed, 1962). In *Simonsiella*, sheets are thought to help cells stay
337 physically anchored in the oral cavity when rapidly shedding epithelial cells slough off
338 (McCowan et al., 1979). We hypothesize that the same may be true for RCUs, which
339 inhabit a similar environment and whose morphology may have undergone convergent
340 evolution due to similar evolutionary pressures. Longitudinal binary fission may be an
341 even more general characteristic that is selected in response to the need to form a secure
342 attachment to a substrate. The segments at the ends of RCUs are often shorter than
343 those closer to the center, suggesting that there may be a mechanism by which the growth
344 of segments is determined by their spatial positioning within an RCU.

CryoTEM images suggested that RCUs are encapsulated by a periodic surface covering, which may be an S-layer or a new crystalline structure. S-layers are self-assembling, crystalline arrays of single proteins or glycoproteins that coat the exterior of some bacteria and archaea (Fagan and Fairweather, 2014; Sleytr et al., 2007). While their exact function varies widely across microorganisms and is often unknown, S-layers are hypothesized to confer beneficial functions given their high metabolic cost (the S-layer comprises up to ~20% of the total protein synthesized by cells), their ubiquity across microbes, and their multiple evolutionary origins (Fagan and Fairweather, 2014; Sleytr et al., 2007). If segments in RCUs correspond to individual cells, production of the periodic surface covering may represent cooperation between cells in RCUs. Cooperative synthesis of a single, shared periodic surface covering by multiple cells could have evolved since close kin (other cells in an RCU) have limited dispersal ability and are therefore situated in close physical proximity. RCUs would benefit from cooperative production of a single periodic surface covering around a population of cells rather than around each individual segment by reducing the surface area required to cover all cells, and such an advantage could even have contributed to selection for aggregation. An additional and not mutually exclusive possibility is that the periodic surface covering may help to maintain the ultrastructure of segments within an RCU, similar to archaea such as *Thermoproteus tenax* (Wildhaber and Baumeister, 1987).

One of the most striking features of RCUs is their pilus-like appendages. At present, there are five characterized classes of pili in Gram-negative bacteria (Chaperone-usher, Curli fibers, F-type, type IV, and type V) and two general types of pili in Gram-positive bacteria (short, thin rods and longer, flexible, hair-like filaments) (Hospenthal et al., 2017; Proft

370 and Baker, 2009); other pilus-like appendages have been documented, such as hami in
371 archaea (Moissl et al., 2005). To the best of our knowledge, characterized bacterial pili
372 all consist of single appendages that exist as independent units. By contrast, the pilus-
373 like appendages that protrude from RCU segments exhibit an unusual architecture
374 involving heterogeneous bundles of parallel filaments that often splay out at the tips.
375 These observations raise the question of whether the RCU appendages represent a novel
376 type of assembly of pilin subunits, or are a completely distinct class of appendages.
377
378 Extensive investigation of hundreds of cryoET tomograms enabled visualization of the
379 structure of many RCU features at high resolution. Future studies of RCUs may benefit
380 from imaging with phase-plate optics that dramatically increase image contrast (Danev
381 and Baumeister, 2017) following specimen preparation methods that thin cells into
382 lamellae by focused ion beam (FIB) milling coupled with scanning electron microscopy
383 (SEM) at cryogenic temperatures (Marko et al., 2007; Wu et al., 2020). Such approaches
384 could enable a comprehensive analysis of the community of RCUs as well as higher
385 resolution structural details of subcellular components of interest via subtomogram
386 averaging.
387
388 The vast majority of microorganisms on Earth lack isolated representatives (Hug et al.,
389 2016). Sequencing-based analyses have proved invaluable in exploring and describing
390 said diversity, yet cannot be used to explore all aspects of the biology of microorganisms.
391 Notable blind spots in our understanding of uncultured organisms include the unique
392 genes and corresponding structural and functional features that have evolved within these
393 lineages. While the use of advanced imaging techniques to visualize microbes can
394 provide insight into the biology of uncultured lineages, a shift toward a more multifaceted

395 approach drawing on many disciplines and techniques will be required to create a
396 comprehensive view of this biological dark matter (Castelle and Banfield, 2018;
397 Ponomarova and Patil, 2015).
398
399
400 **ACKNOWLEDGMENTS**
401 We thank members of the Relman lab for insightful discussion, Katharine Ng, and Brian
402 Yu for assistance in attempts to capture RCUs using laser capture microdissection and
403 microfluidics, respectively, and Melissa Clark for assistance with Gram staining, Michael
404 Schmid for analysis of the periodic surface covering on RCUs, and especially Celeste
405 Parry and colleagues at the U.S. Navy Marine Mammal Program in San Diego, CA for
406 collecting dolphin oral samples. This work was supported by NIH grant P41GM103832
407 (W.C.), a James S. McDonnell Postdoctoral Fellowship (H.S.), and the Thomas C. and
408 Joan M. Merigan Endowment at Stanford University (D.A.R.). K.C.H. and D.A.R. are Chan
409 Zuckerberg Biohub Investigators.
410
411 **AUTHOR CONTRIBUTIONS**
412 Conceptualization: N.K.D, K.C.H., D.A.R.
413 Methodology and investigation: N.K.D., J.G.G.M., H.S., M.M., C.D., G.H.W, B.B., K.C.H,
414 W.C., D.A.R.
415 Formal analysis: N.K.D, J.G.G.M., H.S., C.D.
416 Visualization: N.K.D, J.G.G.M, H.S., C.D.
417 Writing - original draft: The manuscript was mainly written by N.K.D. and J.G.G.M. based
418 on N.K.D.'s original draft, with revisions by all other authors.
419 Writing - review and editing: all authors.

Supervision: K.C.H, W.C., D.A.R.

DECLARATIONS OF INTEREST

The authors declare no competing interests.

FIGURE LEGENDS

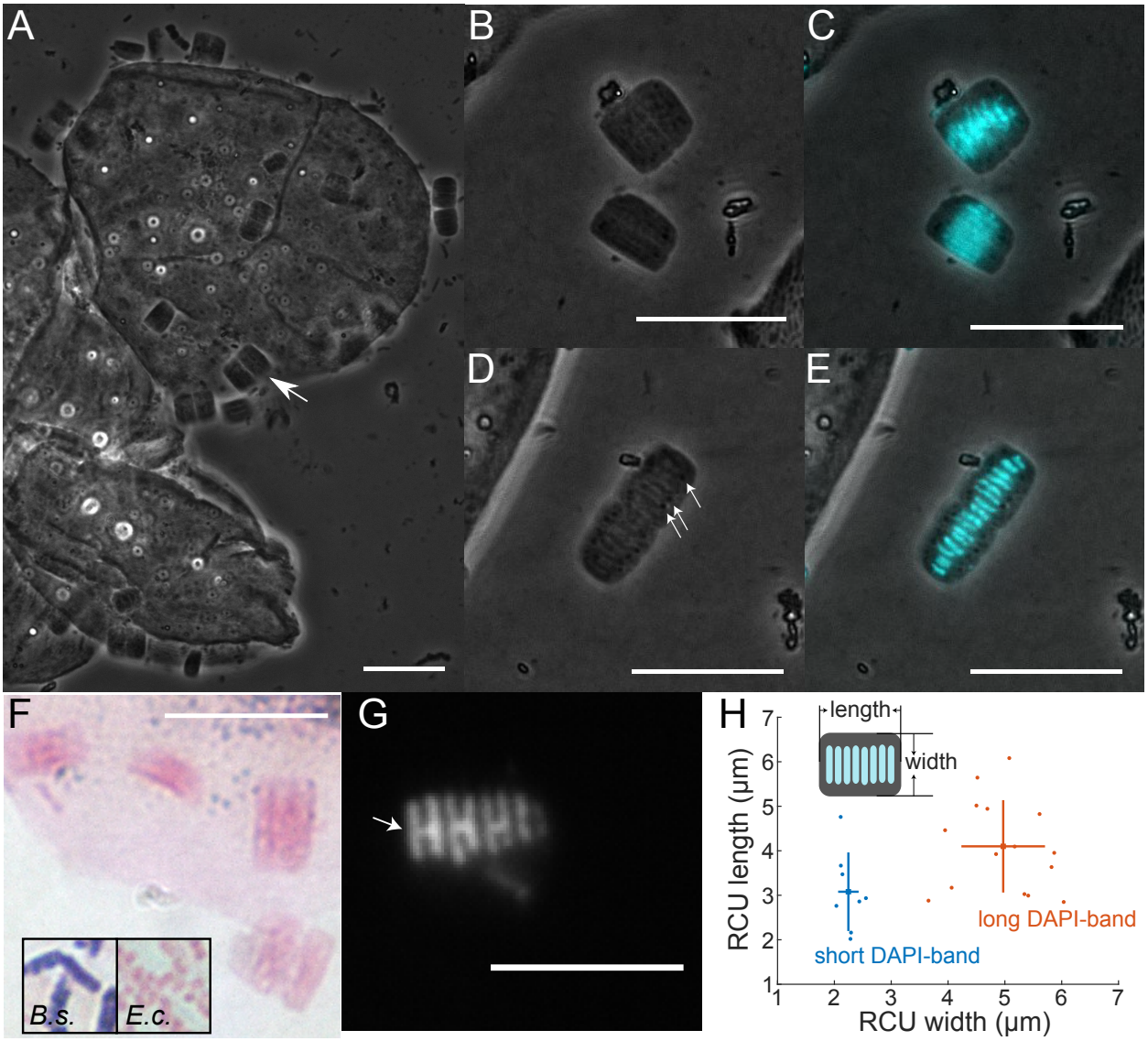

Figure 1. Light microscopy reveals RCUs with multiple morphotypes and distinct DNA banding patterns.

A) Phase-contrast images of RCUs (arrow indicates one example) on the surface of dolphin oral epithelial cells. See also **Movie S1**. Scale bar: 10 μm.

B,C) Some RCUs exhibited long bands of DAPI fluorescence. Phase-contrast image is shown in (B), with fluorescence overlay in cyan in (C). Scale bars: 10 μm.

D,E) Other RCUs exhibited shorter DAPI bands. Phase-contrast image is shown in (D), with fluorescence overlay in cyan in (E). Dark spots (arrowheads) were

434 organized in lines perpendicular to DAPI-stained bands. DAPI-stained bands
435 appeared to be organized in pairs. Scale bars: 10 μm.
436 F) Gram-stained RCUs display Gram-negative characteristics. Inset: Gram-stained
437 *Bacillus subtilis* (*B.s.*, Gram-positive) and *Escherichia coli* (*E.c.*, Gram-negative).
438 Scale bar: 10 μm.
439 G) Neighboring DNA band pairs in an RCU form “H”-like shapes (white arrow), likely
440 because the DNA bands are nucleoids segregating in a cell undergoing
441 longitudinal division. Scale bar: 10 μm.
442 H) The two RCU morphotypes have distinct distributions of length and width.
443

Figure 2. 16S rRNA amplicon sequencing of dolphin oral samples indicates that RCUs are not affiliated with the *Simonsiella* genus. Three dolphin oral samples visually confirmed to contain RCUs were subjected to 16S rRNA gene amplicon sequencing. All detected ASVs are shown. Each row shows ASVs with their lowest

450 taxonomic assignment (level denoted by prefix such as “s__” for species). Taxonomic
451 groups are color-coded by phylum, with the polyphyletic Proteobacteria group shown at
452 the level of Class.

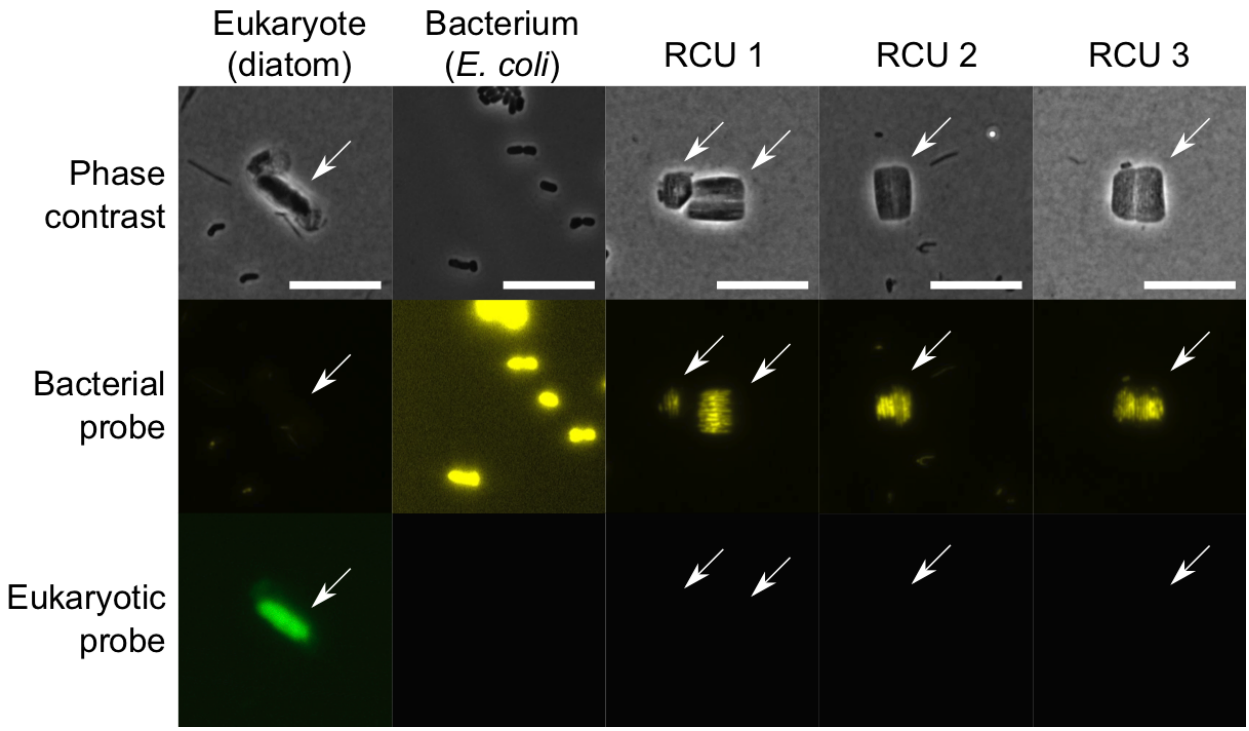

Figure 3. Fluorescence in situ hybridization indicates that RCUs are bacterial rather than eukaryotic. Bacterial probe Eub-338 was labeled with AlexaFluor-488 and eukaryotic probe Euk-1209 was labeled with AlexaFluor-660. Top: phase-contrast images; middle, bottom: fluorescence images from bacterial and eukaryotic probes, respectively. Arrows indicate the relevant cells in non-axenic samples. The first column is a cell of the marine diatom *Skeletonema costatum* grown in non-axenic culture. The second column is axenic *Escherichia coli* cells. The last three columns are RCUs obtained directly from dolphin oral swabs. All RCUs were labeled with the bacterial probe, while the eukaryotic probe only labeled the marine diatom. Scale bars: 10 μm.

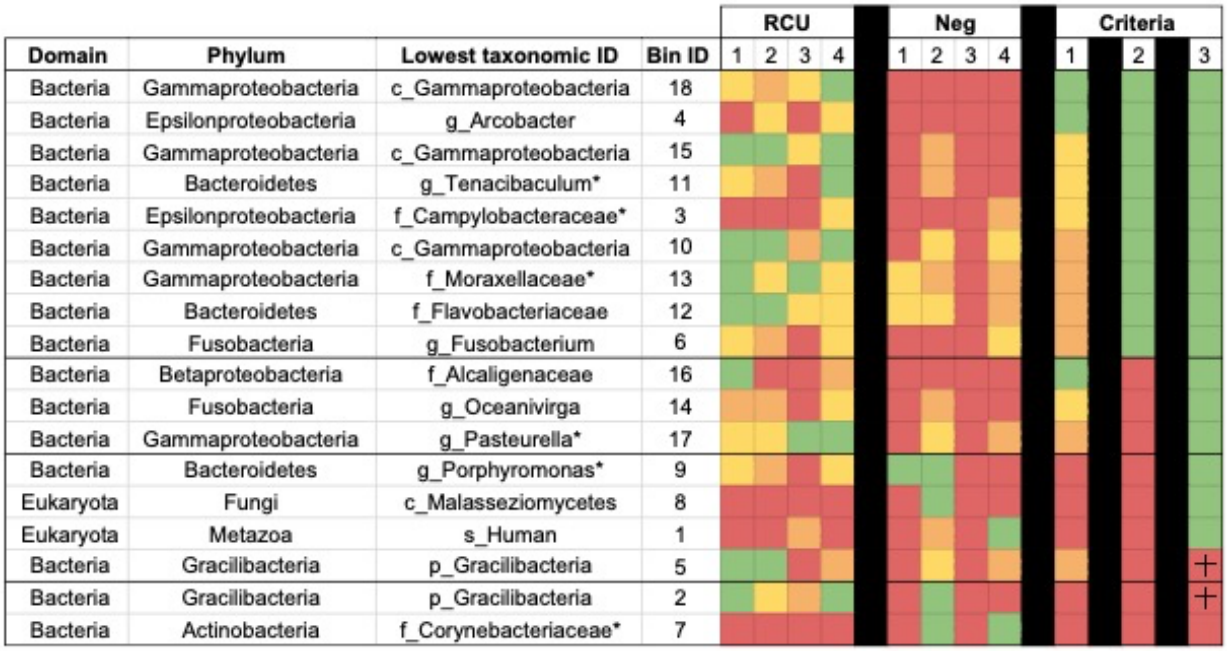

Figure 4. Insights into RCU identity. The 18 bins recovered from MDA and sequencing of RCU samples collected via micromanipulator, along with the Phylum from which they are inferred to derive. For lowest taxonomic identity achieved (or Class, in the case of the polyphyletic Proteobacteria), an asterisk (*) denotes lower confidence in the assignment (Methods). The RCU panel shows relative abundances of bins in each of the four samples that contained RCUs based on visualization, color-coded as follows: green: ≥5%, yellow: ≥1% and <5%, orange: >0% and <1%, red: 0%. The negative-control panel (Neg) presents the same information for each of the samples that did not appear to contain RCUs. Criteria for gauging the likelihood of a bin deriving from the RCUs are shown. (1) Was the bin ever present in negative controls (green = no, yellow = yes but never ≥1% relative abundance, orange = yes but never ≥5%, red = yes and at least once ≥5%). (2) Of ASVs shared between the three dolphin oral samples that produced the RCU panel, was any shared ASV a putative candidate for the bin? For example, bin 14 was identified as from the family Oceanivirga; was any shared ASV a member of Oceanivirga? (green = yes, red = no). (3) Are members of a given phylum known to be Gram-negative (green

479 = yes, red = no)? A “†” denotes that members of the Gracilibacteria phylum are inferred
480 not to be Gram-negative from genomic studies (although they are not necessarily Gram-
481 positive) (Meheust et al., 2019). Highest likelihood candidates are green for all three
482 criteria.

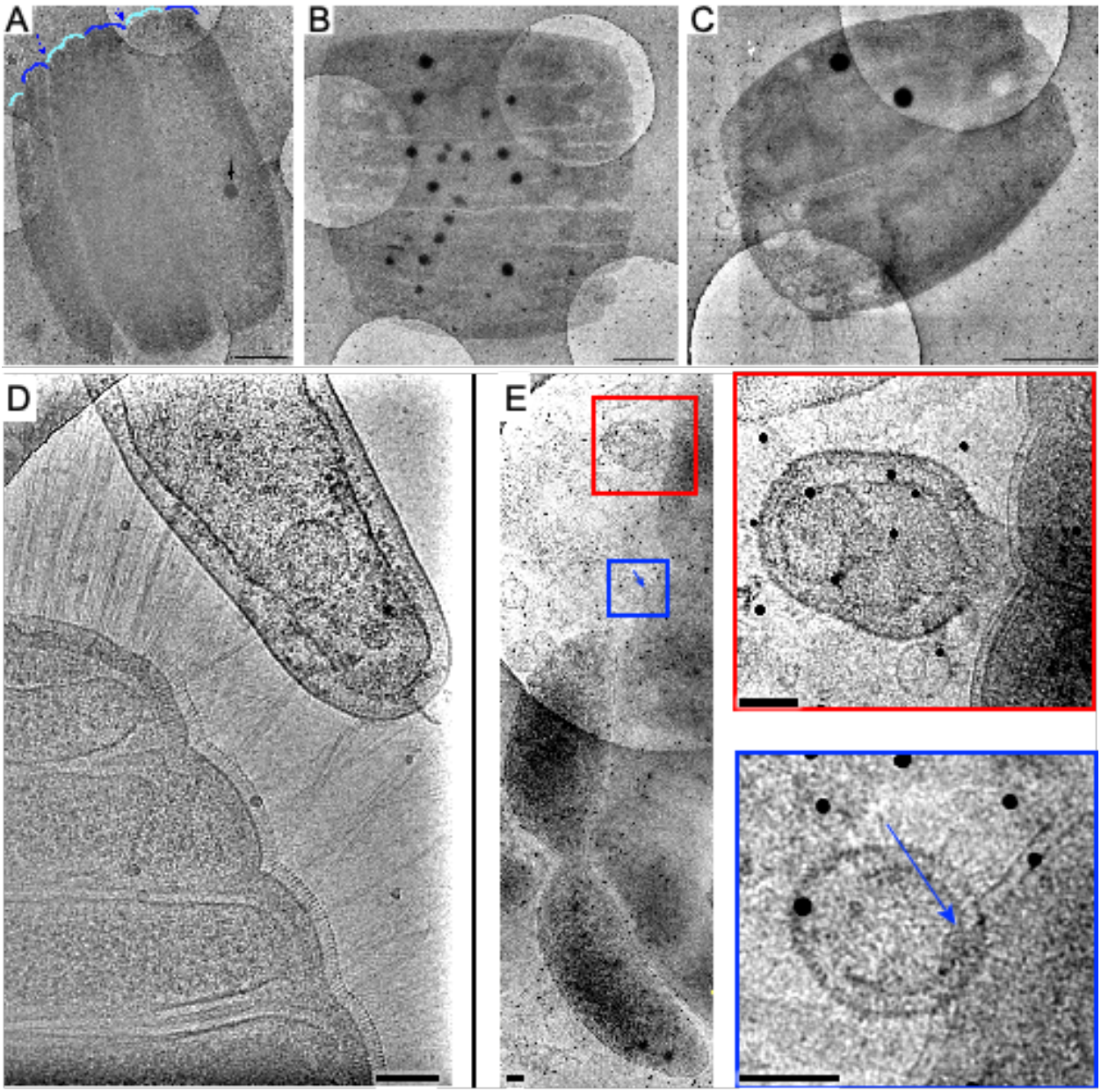

Figure 5. CryoTEM demonstrates that RCUs consist of multiple parallel, likely paired segments and are often near other cells. Band-pass filtered and denoised low magnification (A) and images or montage cryoTEM images of RCUs on an R2/2 holey carbon TEM grid (B-E). In (A), pairs of segments are highlighted with alternating shades of blue, and sharp indentations between groups of segments are denoted with blue arrows. Dense spheroidal objects inside RCUs may be storage granules or lipid droplets (black arrow). (D, E) RCUs were often in close physical proximity to other cells. In (E), an

491 apparent small indentation (blue arrow) in the RCU periodic surface covering overlaps
492 with a non-RCU cell. Small dark spots (15 nm) in (C-E) are gold fiducial particles for
493 cryoET experiments (white arrow). Scale bars: 1 μm.

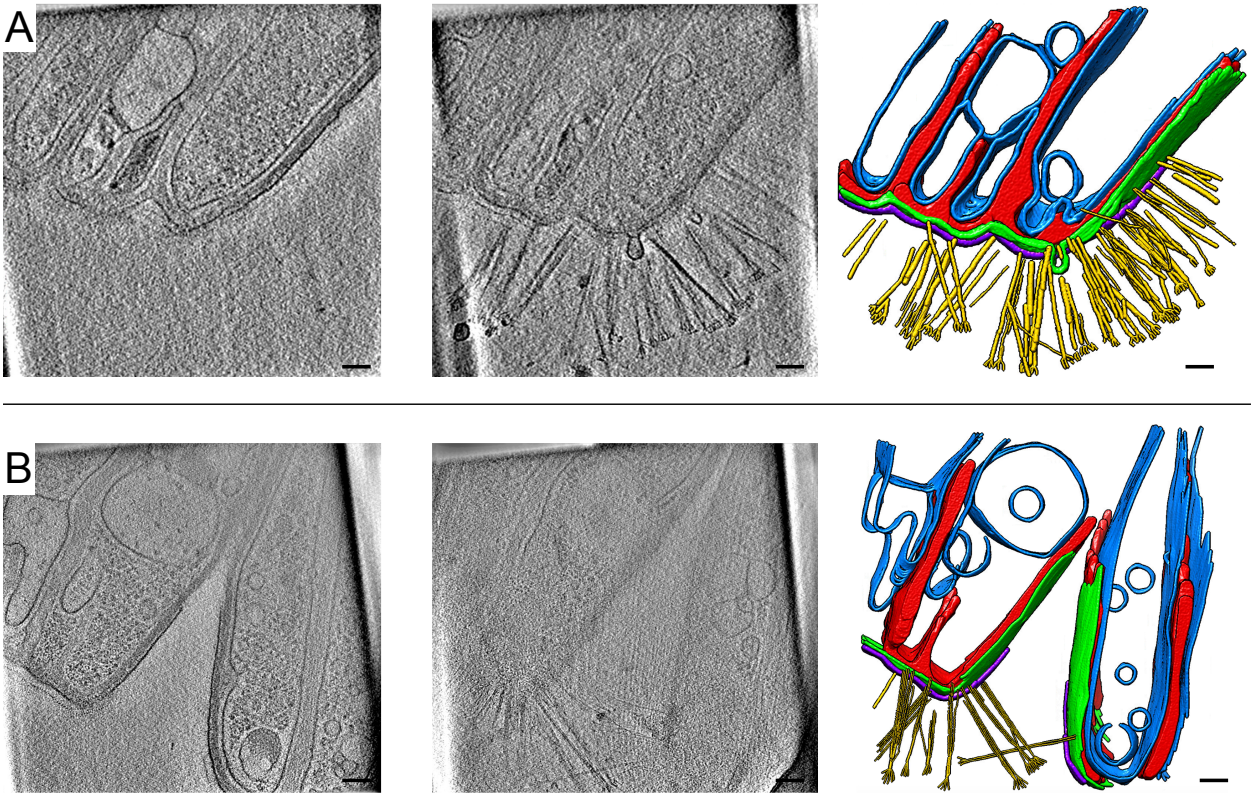

Figure 6. CryoET reveals the three-dimensional architecture of RCU components. A, B) Examples of ~3 nm-thick slices at two different depths (left and center) from two representative RCU tomograms, and corresponding manual annotations of cellular features (right). The tomograms are thick (~500-600 nm) and as such, pilus-like appendages (yellow) were visible only at a certain depth within the tomographic volume density of the RCU. See also **Movies S2 and S3.** Blue, inner membranes; purple, periodic surface covering; green, outer membrane; red, matrix. Scale bars: 100 nm.

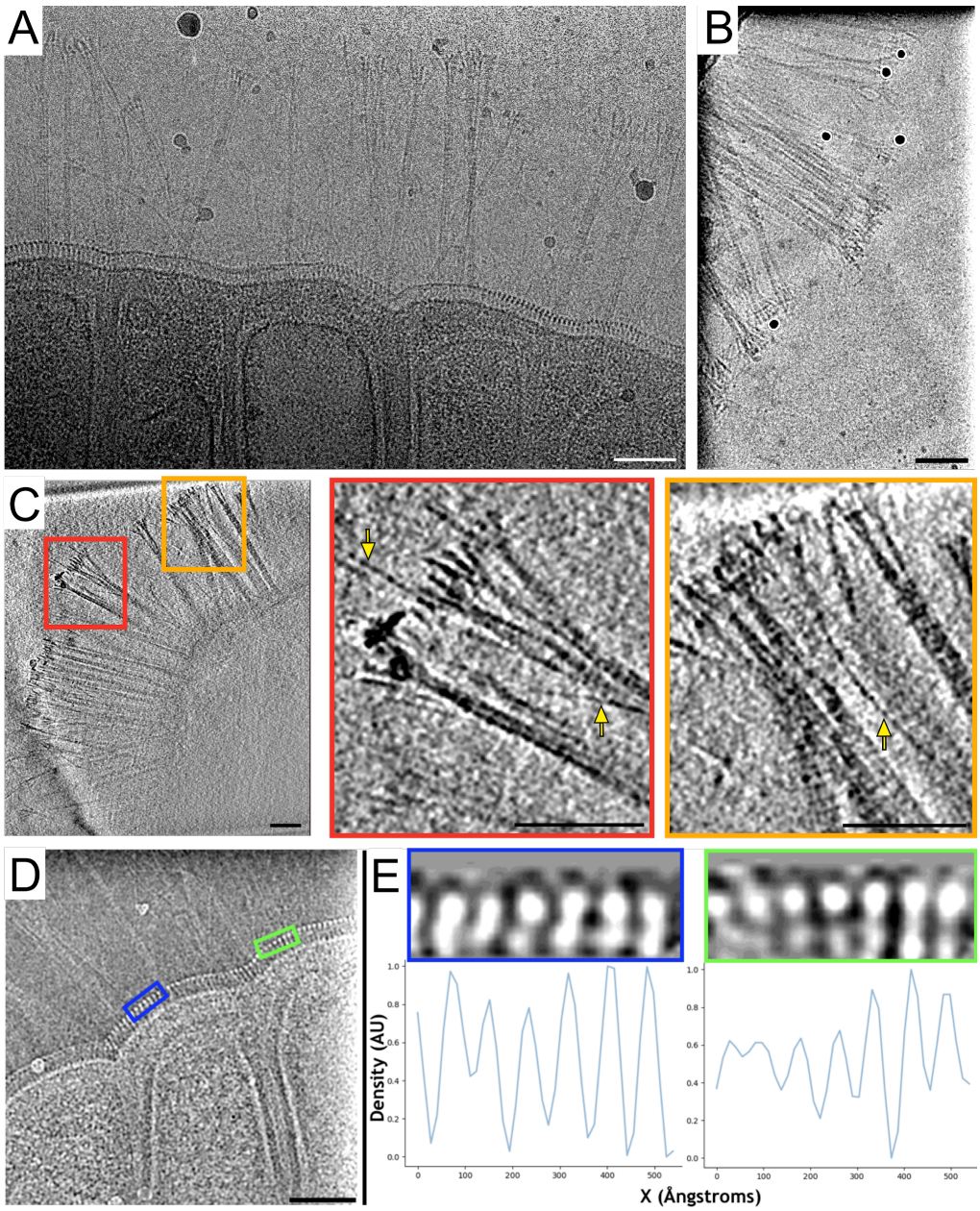

Figure 7. RCU surface features include appendages in heterogeneous bundles that splay out at the tips and a periodic surface covering around the entire RCU.

A) A single cryoTEM image.

B,C) CryoET tomographic slices (~3-nm thick) of an RCU. Red and orange boxes in (C) are magnified views of bundles of appendages, and yellow arrows denote thin, single appendages. Scale bars: 100 nm.

509 D) Representative 2D cryoTEM image at the edge of an RCU showing a periodic
510 surface covering.
511 E) Line density profiles along selected regions from the image in (D) show that the
512 spacing of the repetitive features is ~7-9 nm along a direction parallel to the RCU
513 membrane. Scale bar: 100 nm.

514 **STAR METHODS**

515

516 ***RESOURCE AVAILABILITY***

517

518 **Lead contact**

519 Further information and requests for resources and reagents should be directed to and
520 will be fulfilled by the Lead Contact, David Relman (relman@stanford.edu).

521

522 **Materials availability**

523 This study did not generate new unique reagents.

524

525 **Data and code availability**

526 Raw single-cell sequence reads and 16S rRNA sequence reads are available through
527 NCBI BioProject database PRJNA174530 with BioSample identifiers SAMN19013666,
528 SAMN19013667, and SAMN19013668.

529

530 ***EXPERIMENTAL MODEL AND SUBJECT DETAILS***

531 Oral swab samples were obtained from bottlenose dolphins (*Tursiops truncatus*)
532 managed by the U.S. Navy Marine Mammal Program (MMP) Biosciences Division, Space
533 and Naval Warfare Systems Center Pacific, San Diego, USA. The earliest sample
534 containing RCUs was collected on April 1, 2012 and the latest on February 22, 2018.
535 Samples were obtained by swabbing the roof of the mouth, the tongue, and between the
536 tongue and mandible. Swabs were obtained using sterile foam Catch-All sample
537 collection swabs (Epicenter, WI, Cat. #QEC091H). The swabbing protocol adhered to the
538 guidelines described in the CRC Handbook of Marine Mammal Medicine. The MMP is

539 accredited by the Association for Assessment and Accreditation of Laboratory Animal
540 Care (AAALAC) International and adheres to the national standards of the United States
541 Public Health Service Policy on the Humane Care and Use of Laboratory Animals and
542 the Animal Welfare Act. As required by the U.S. Department of Defense, the MMP's
543 animal care and use program is routinely reviewed by an Institutional Animal Care and
544 Use Committee (IACUC) and by the U.S. Navy Bureau of Medicine and Surgery. The
545 animal use and care protocol for MMP dolphins in support of this study was approved by
546 the MMP's IACUC and the Navy's Bureau of Medicine and Surgery (IACUC #92-2010,
547 BUMED NRD-681). Samples were collected during 2012 and 2018.
548
549 ***METHOD DETAILS***
550
551 **Microscopy sample preparation**
552 To separate cells from swabs, swabs were immersed in 1X PBS (~50-100 µL, depending
553 on cell density) in microcentrifuge tubes. Tubes were vortexed vigorously for ~10 s and
554 lightly centrifuged to remove liquid from tube caps. The resulting solution was used for
555 microscopy.
556
557 **Light microscopy**
558 Approximately 1 µL of cell solution in PBS was spotted onto an agarose pad (1% agarose
559 in PBS) and imaged with an Eclipse Ti-E inverted microscope with a 100X (NA: 1.4)
560 objective (Nikon, Tokyo, Japan). To determine DNA localization, cells were stained with
561 DAPI at a final concentration of 0.5 µg/mL prior to imaging for 5 min and imaged using
562 emission/excitation spectra of 340/488 nm.
563

564 **Gram staining**

565 Gram staining was performed using a PREVI COLOR GRAM automated machine
566 (bioMerieux, Marcy-l'Étoile, France).

567

568 **RCU cryofixation and cryoEM/ET data acquisition**

569 A solution of cells in PBS (2.5 µL) was applied to glow-discharged 200-mesh copper,
570 holey-carbon Quantifoil grids (Quantifoil, Großlöbichau, Germany, Cat. #Q2100CR1) with
571 holey carbon or gold GridFinder Quantifoil grids (Quantifoil, Großlöbichau, Germany, Cat.
572 #LFH2100AR2), followed by application of 2 µL of 15-nm gold fiducial solution to both
573 sides of each grid. Grids were blotted for 5 s and plunge-frozen in liquid ethane cooled
574 by liquid nitrogen to approximately -195 °C using an EM GP Plunge Freezer (Leica,
575 Wetzlar, Germany).

576

577 Samples were loaded into one of two microscopes: a Titan Krios G3 operated at 300 kV
578 with an energy filter (20-eV slit width), or a Titan Krios G4 operated at 300 kV without an
579 energy filter. Both microscopes were equipped with a K2 Summit direct electron detection
580 device (Gatan, Pleasanton, USA) used to record micrographs. Data were acquired semi-
581 automatically in counting mode using SerialEM (Mastronarde, 2003). CryoEM/ET imaging
582 parameters are provided in **Table S5**.

583

584 **CryoEM/ET data processing**

585 Montages were blended and binned 4-fold or greater using IMOD's "blendmont" algorithm
586 (Kremer et al., 1996) and normalized, band-pass filtered, rotated, and cropped for display
587 purposes using EMAN2 (Tang et al., 2007). Fifteen out of sixteen tilt series were suitable
588 for tomographic reconstruction in IMOD. Tilt series with sampling at 7.5 Å/pixel were

down-sampled by 2-fold and those with sampling at 3.48 Å/pixel or 3.75 Å/pixel were down-sampled by 4-fold. Images with artifacts such as excessive charging, drifting, large ice contamination creeping in at high tilts, or excessive thickness at high tilt were excluded from 12 of the tilt series prior to manual gold-fiducial-based alignment; up to 13 images were removed out of the 41 images in the original raw tilt series. Tomograms were reconstructed using standard weighted back-projection and a SIRT-like filter (mimicking 16 iterations) and were band-pass-filtered and further binned by 2-fold in most cases for feature annotation, segmentation, movie production, and other display purposes. Tomogram thickness was estimated by visually identifying the smallest and largest *z*-slices with visible RCU or ice contamination densities and converting the number of slices to nanometers. Subtomogram averaging was attempted using EMAN2 (Galaz-Montoya et al., 2015; Galaz-Montoya et al., 2016) for globular densities suspected to be ribosomes, matrix densities under the outer membrane, patches of the periodic surface covering, and regions of pilus-like appendages, but no interpretable structures with resolution better than ~50 Å were attained. The ranges of thickness and length for the pilus-like appendages were derived by visually scanning the slices in the tomograms for the thinnest individual filaments and thickest bundles perceptible to the naked eye, and measuring their dimensions in binned-by-4 tomographic slices using the measuring tape tool of EMAN2's `e2display.py`. The repeat distance of the periodic surface covering was measured manually in a similar fashion as the pilus-like appendages from tomographic slices, with ~10-20 measurements from each of three tomograms displaying at least small regions where the repeat was discernible. This quantification yielded a range between ~6 and 10 nm, suggesting that either the layer components are flexible or that the underlying structure can yield different apparent distances between its subunits depending on the angle at which it is sliced. Additionally, regions showing the pattern

614 much more clearly in higher-magnification montage two-dimensional projection images
615 were cropped out, rotated to lie in a horizontal plane, filtered, and masked to compute
616 line-density profiles parallel to the outer membrane. This strategy allowed measurement
617 of the distance between consecutive peaks and/or consecutive valleys, yielding values
618 between ~7-9 nm.
619
620 We initially carried out tomographic annotation of three features (periodic surface
621 covering, lipid membranes, and pilus-like appendages) for three tomograms using
622 EMAN2's semi-automated two-dimensional neural network-based pipeline (Chen et al.,
623 2017) and performed manual clean-up of false positives in UCSF Chimera (Pettersen et
624 al., 2004). The output annotation probability maps from EMAN2 were turned into
625 segmentations by applying a threshold that was visually satisfying and multiplying the
626 contrast-reversed tomograms by the thresholded annotation map. The segmentations
627 were low-pass-filtered with EMAN2 to smooth out noise. However, since the complexity
628 of subcellular structures was not captured by the semi-automated annotations, we applied
629 a similar process to generate segmentations of five features (pilus-like appendages,
630 periodic surface covering, outer membrane, matrix, and inner membranes) using manual
631 annotations performed with IMOD. Snapshots for **Figure 6** displaying RCU features in
632 color as well as **Movies S2 and 3** showing segmentation results were produced with
633 UCSF Chimera.
634
635 **Fluorescence *in situ* Hybridization**
636 Cell cultures of axenic *Escherichia coli* MG1655 and non-axenic *Skeletonema costatum*
637 LB 2308 (UTEX Culture Collection of Algae at the University of Texas at Austin, Austin,
638 TX, USA) were prepared as controls. *E. coli* was cultured in LB broth and grown at 37 °C,

639 and *S. costatum* was cultured in Erdschreiber's Medium at 20 °C with a ~12 h light and
640 ~12 h dark cycle.
641
642 Probes Bact-338 and Euk-1209 were used to identify bacterial and eukaryotic cells,
643 respectively. Probes were ordered from Integrated DNA Technologies (Coralville, USA)
644 with HPLC purification as follows: Euk-1209 labeled with Alexa Fluor 660
645 (GGGCATCACAGACCTG/3AlexF660N) and Bact338 labeled with Alexa Fluor 488
646 (GCTGCCTCCCGTAGGAGT/3AlexF488N).
647
648 Cells from controls and RCUs were collected in microcentrifuge tubes. To ensure
649 sufficient biomass from dolphin oral swabs, cells from four swabs were condensed into a
650 single tube. The FISH protocol was adapted from (Skinner et al., 2013). Cells were fixed
651 in 1 mL of 3.7% formaldehyde solution (800 µL DEPC-treated water, 100 µL 10X PBS,
652 100 µL 37% formaldehyde) for 30 min with gentle shaking at 700 rpm. Cells were then
653 washed twice in 1 mL of 1X PBS, and permeabilized in a mixture of 300 µL DEPC-treated
654 water and 700 µL 200-proof ethanol with gentle shaking at 700 rpm for 2 h. Probes were
655 added to 50 µL of 40% hybridization solution (5 mL DEPC-water, 1 g dextran sulfate, 3.53
656 mL formamide, 1 mL 2X SSC, brought to a total volume of 10 mL with DEPC-treated
657 water) to a final concentration of 1 µM per probe set. Cells were incubated overnight in
658 50 µL of hybridization buffer with FISH probes at 30 °C. Cells were washed twice using a
659 wash solution (2 mL 20X SSC buffer, 7.06 mL formamide, 10.94 mL DEPC-treated water)
660 and resuspended in 2X SSC buffer.
661
662 Imaging data were processed using FIJI v. 2.0.0 (Schindelin et al., 2012).
663

664 **16S rRNA amplicon sequencing and processing**

665 Three dolphin oral samples that were confirmed to contain RCUs with long DAPI bands
666 were selected for 16S rRNA amplicon sequencing. DNA was extracted using the QiaAMP
667 DNA Mini Kit (Qiagen, Valencia, USA, Cat. #51304) as described in (Bik et al., 2016). The
668 V4 region of the 16S rRNA gene was PCR-amplified in triplicate using barcoded 515F
669 forward primers and the 806RB reverse primer. PCR was performed in triplicate using the
670 5 Prime Hot Master Mix (Quantabio, Beverly, USA Cat. #2200410). PCR products were
671 purified using the UltraClean 96 PCR Cleanup Kit (Qiagen, Valencia, USA, Cat. #12596-
672 4) and pooled in equimolar ratio following DNA quantification using the Quant-iT dsDNA
673 assay kit (Thermo Fisher Scientific, Waltham, USA, Cat. #Q33120). Finally, DNA was run
674 through a Zymo Clean and Concentrate Spin Column (Zymo Research Corporation,
675 Irvine, USA, Cat. #D4029) and further purified using the QIAquick gel extraction kit
676 (Qiagen, Hilden, Germany, Cat. #28704). Amplicons were sequenced in a single 2x250
677 nt Illumina HiSeq 2500 lane at the W. M. Keck Center for Comparative Functional
678 Genomics at the University of Illinois, Urbana-Champaign.

679

680 Demultiplexing was performed using QIIME v. 1.9.1 (Caporaso et al., 2010). Amplicon
681 sequencing variants (ASVs) were inferred using DADA2 v. 1.6.0 (Callahan et al., 2016),
682 following guidelines in the "Big Data Workflow"
683 (https://benjjneb.github.io/dada2/bigdata_paired.html). Forward and reverse reads were
684 trimmed to 245 nt and 200 nt, respectively. ASVs were inferred separately for forward
685 and reverse reads using lane-specific error-rate profiles, and paired reads were merged.
686 The “removeBimeraDenovo” function in DADA2 was used to identify and remove
687 chimeras from sample datasets and taxonomic assignments were created using the
688 DADA2 "assignTaxonomy" and "assignSpecies" functions, using RDP training set 16 as

689 a reference database (Cole et al., 2014). Additional stringent filtering of amplicons was
690 performed, using VSEARCH v. 2.8.0 to remove chimeras (Rognes et al., 2016), BLAST
691 v. 2.7.1 (Altschul et al., 1990) and the 16S rRNA gene set in (Schulz et al., 2017) to retain
692 only bacterial 16S rRNA genes (e.g. to remove chloroplast 16S rRNA genes), scripts to
693 remove abnormally short ASVs (<1200 bp) or those that contained N's or X's, and
694 Decontam v. 0.99.3 to remove contaminant ASVs (Davis et al., 2018). This pipeline
695 yielded a total of 394 taxa and 991,592 reads across the three samples.
696

697 **Single-cell genomics**

698 To obtain candidate identities for RCUs, we employed a single-cell genomics approach.
699 To limit contamination by foreign DNA, reagents, tubes, and PBS were treated with 11.4
700 J/cm^2 of ultraviolet light following the guidelines in (Woyke et al., 2011). RCUs were
701 visualized with an Olympus IX70 inverted microscope (Olympus, Waltham, USA) with a
702 40X objective and Hoffman modulation optics. An Eppendorf TransferMan
703 micromanipulator (Eppendorf, Hamburg, Germany, Cat. #5193000020) with a SAS-10
704 microinjector was used to capture RCUs with Polar Body Biopsy Micropipettes (30°
705 angled, beveled, and polished with an inner diameter of 13-15 μm) (Cooper Surgical,
706 Målov, Denmark, Cat. #MPB-BP-30). After an RCU or chain of RCUs was acquired, the
707 micropipette tip was transferred to a collection tube containing 1X PBS and crushed into
708 the tube to ensure the RCU(s) was deposited in the tube; this precaution was adopted
709 because RCUs frequently stuck to the glass micropipettes and could not be dislodged.
710 No dolphin cells were captured, although cell-free DNA and small, non-target cells from
711 the sample were likely acquired as contaminants along with RCUs based on the
712 propensity for attachment of other species (**Figure 5E**). Four tubes of RCUs were
713 collected (sample names RCU1, RCU2, RCU3, RCU4), along with four negative-control

714 tubes (NEG1, NEG2, NEG3, NEG4). Negative controls consisted of draws of PBS from
715 the same sample that did not contain any visible cells, and were otherwise treated
716 identically to RCU-containing samples.
717
718 DNA from each tube was amplified via multiple displacement amplification (MDA) using
719 the Repli-g single-cell kit (Qiagen, Hilden, Germany, Cat. #150343) according to the
720 manufacturer's protocol. DNA was purified using a Zymo Clean and Concentrate Spin
721 Column (Zymo Research Corporation, Irvine, USA, Cat. #D4013) and libraries were
722 prepared using the Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, USA, Cat.
723 #KK8504) at the W.M. Keck Center for Comparative Functional Genomics at the
724 University of Illinois, Urbana-Champaign. The eight libraries were sequenced using the
725 Illumina MiSeq 2x250 nt P2 V2 platform. RCU samples RCU1, RCU2, NEG1, and NEG2
726 were pooled and sequenced across a single lane that produced 11,371,243 read pairs,
727 and samples RCU3, RCU4, NEG3, and NEG4 were pooled and sequenced across 1.5
728 lanes, collectively producing 19,615,690 read pairs. Sequencing adaptors were
729 computationally removed at the Keck Center.
730
731 Reads from all eight samples were co-assembled using SPAdes v. 3.11.1 (Bankevich et
732 al., 2012) with the single cell (–sc) and careful (–careful) modes specified. A total of
733 61,973,866 read pairs were used for assembly, resulting in 1,406 scaffolds ≥5 kbp long
734 with a total length of 17,438,233 bp and an N50 of 14,592 for scaffolds ≥5 kbp long.
735 Protein coding genes were identified using Prodigal v. 2.6.2 (Hyatt et al., 2010). Per
736 scaffold average coverage was calculated by mapping reads per sample against the co-
737 assembly using bowtie2 v. 2.2.4 (Langmead and Salzberg, 2012), using the samtools v.

738 1.6 depth function (Li et al., 2009) to calculate per-base read coverage, and a custom
739 script to calculate average per-base read coverage per scaffold.
740
741 To determine the taxonomic identity of sequenced cells, we employed a genome-resolved
742 approach. Assignment of scaffolds to genome bins was performed using the
743 tetranucleotide frequencies of all scaffolds ≥5 kbp long over windows of 5 kbp, as
744 described in (Dick et al., 2009). Results were computed and visualized using the
745 Databionics ESOM Tools software v. 1.1 (Ultsch and Mörchen, 2005), leading to the
746 reconstruction of 18 genome bins (**Figure S3**). To refine bins, we removed scaffolds for
747 which <50% of keys were assigned to the bin. Scaffolds <5 kbp long were not binned.
748 The completeness and contamination per bin were assessed using CheckM v. 1.0.7
749 (Parks et al., 2015). To evaluate how representative binning was of the genomes that
750 were sequenced, we estimated the number of prokaryotic genomes expected to be
751 recovered by searching the metagenome assembly for a set of 16 bacterial single copy
752 genes (bSCGs) assumed to be present in every genome in a single copy (Hug et al.,
753 2013). The median number of each bSCG was 10, suggesting ~10 prokaryotic genomes
754 were represented in our sequencing dataset.
755
756 Taxonomic identification of bins posed a challenge since 16S/18S rRNA genes were not
757 reliably amplified/sequenced/assembled, and genomes were partial with few
758 phylogenetically informative bacterial single copy genes (Hug et al., 2013) present in the
759 dataset. Hence, we used BLAST v. 2.2.30 (Altschul et al., 1990) to query all protein coding
760 genes from each genome against the NCBI non-redundant protein database using an e-
761 value of 10^{-10} and taxonomic assignments were made based on the closest protein match.
762 Genome bin taxonomic assignments were considered highly likely if ≥50% of the top blast

763 hits originated from a single taxon and were considered to be plausible if <50% but ≥33%
764 of the top blast hits originated from a single taxon.
765
766 There are numerous approaches by which one could assess whether a bin is "present" in
767 a sample, each with largely arbitrary thresholds. We focused on the relative abundance
768 of each bin per sample (**Table S4**) as it accounts for the length of each bin and allows for
769 comparisons between samples with different numbers of read pairs (Dudek et al., 2017).
770

771 **Attempt at culturing RCUs**

772 Two dolphin oral samples confirmed to contain RCUs were selected for culturing efforts.
773 For each sample, one milliliter of sterile PBS was added to a 1.5-mL Eppendorf tube
774 containing the oral swab sample. Six hundred microliters of each sample were used to
775 inoculate 3 mL of BSTYS (Kuhn et al., 1978) (2.75% w/v Tryptic Soy Broth, 0.4% w/v
776 yeast extract, 1.5% w/v agar, 10% bovine serum), SHI (Tian et al., 2010), or mSHI media
777 (SHI supplemented with 0.9 g/L NaCl, 2.5 g/L K_2PO_4, 0.84 g/L $NaHCO_3$, 0.17 g/L $CaCl_2$,
778 0.04 g/L $MgCl_2$ * $6H_2O$, and 5 g/L of dextrose), in liquid form and on thin layer agar plates.
779 BSTYS was selected for its use in successfully culturing bacteria (*Simonsiella* specifically)
780 from the oral cavities of various mammals (Kuhn et al., 1978). SHI was selected because
781 this medium was designed to sustain high diversity communities derived from the human
782 oral microflora. mSHI (modified-SHI) was included as a higher-salinity version of SHI in
783 an attempt to further mimic the conditions that might be found in the oral cavity of dolphins.
784 Inoculation was repeated under anaerobic conditions in an anaerobic chamber (COY Lab
785 Products, Grass Lake, USA); note that all samples were unavoidably exposed to
786 atmospheric oxygen prior to culturing. Cultures were incubated at 37 °C to mimic the body

787 temperature of dolphins. No RCUs were detected on solid media or in liquid media by
788 visual screening under a microscope after ~24, ~48, ~72, and ~96 h.
789

790 ***QUANTIFICATION AND STATISTICAL ANALYSIS***

791 Detailed descriptions of the quantitative methods used in this paper can be found in the
792 Results and Method Details sections. Briefly, these descriptions include the methods
793 used for DNA extraction, sequencing, read quality filtering and analysis, and microscopy
794 data acquisition.

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